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Citation

Ping, Liyan, Yilin Wu, Basarab G. Hosu, Jay X. Tang, and Howard C. Berg. 2014. "Osmotic Pressure in a Bacterial Swarm." *Biophysical Journal* 107, no. 4: 871–878. doi:10.1016/j.bpj.2014.05.052.

Published Version

doi:10.1016/j.bpj.2014.05.052

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Osmotic pressure in a bacterial swarm

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Runing Title: Swarm Osmolarity

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Keywords: *Escherichia coli*, bacterial motility, liposome sensor

10 ABSTRACT Using *Escherichia coli* as a model organism, we have studied how water is
11 recruited by a bacterial swarm. Previous analysis of trajectories of small air bubbles revealed a
12 stream of fluid flowing in a clockwise sense ahead of the swarm. A companion study suggested
13 that water moves out of the agar into the swarm in a narrow region centered $\sim 30 \mu\text{m}$ from the
14 leading edge of the swarm and then back into the agar (at a smaller rate) in a region centered
15 $\sim 120 \mu\text{m}$ back from the leading edge. Presumably, these flows are driven by changes in
16 osmolarity. Here, we utilized green/red fluorescent liposomes as reporters of osmolarity in order
17 to verify this hypothesis. The stream of fluid that flows in front of the swarm contains osmolytes.
18 Two distinct regions are observed inside the swarm near its leading edge: an outer high-
19 osmolarity band $\sim 30 \text{ mOsm}$ higher than the agar baseline and an inner low-osmolarity band
20 isotonic or slightly hypotonic to the agar baseline. This profile supports the fluid-flow model
21 derived from the drift of air bubbles and provides new insights into water maintenance in
22 bacterial swarms. High osmotic pressure at the leading edge of the swarm extracts water from
23 the underlying agar and promotes motility. The osmolyte is of high molecular weight, probably
24 lipopolysaccharide.

27 INTRODUCTION

28 When some bacteria grow in a rich medium on the surface of moist agar, cells elongate,
 29 multinucleate, grow flagella, and swarm across the surface in coordinated packs (1-3).
 30 Swarming promotes invasiveness of bacterial pathogens, as has been confirmed in a wide range
 31 of clinical isolates, including *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Bacillus cereus*,
 32 *Salmonella enterica*, etc.(4-7). In 1994, Harshey and Matsuyama discovered that strains of
 33 *Escherichia coli* K-12 swarm on Eiken agar (from Japan) rather than on Difco agar, presumably
 34 because Eiken agar is more wettable (8). Many bacteria produce surfactants as they swarm,
 35 which influences patterns of expansion (2, 3). However, these surfactants are not essential; in
 36 particular, there is no indication that *E. coli* produces surfactants (9). Absence of surfactants
 37 simplifies efforts to establish the role played in swarm expansion by osmotic flow.

38 An *E. coli* swarm consists of an actively expanding rim of cells followed by a relatively
 39 inactive interior (10, 11). The cells swim in a thin film of fluid, the upper surface of which is
 40 stationary (12). This film extends some 10-20 μm ahead of the leading edge of the swarm.
 41 Tracking of microbubbles prepared from the surfactant Span 83 indicated that this region of fluid
 42 streams clockwise in front of the swarm at a rate about 3 times that of the swarm advance (12).
 43 This chiral flow is driven by the counterclockwise rotation of the flagella of cells stalled at the
 44 edge of the swarm (12, 13). Patterns of flow of fluid within the swarm also were inferred from
 45 the motion of microbubbles. The data could be fit by a model in which a large amount of fluid
 46 moved from the agar into the swarm near its leading edge, while a smaller amount moved from
 47 the swarm back into the agar $\sim 100 \mu\text{m}$ further behind (10). The bulk fluid in a swarm provides

the environment for flagellar rotation and for transportation of nutrients and signaling molecules.

The only source for this fluid is the underlying agar. One of the intriguing questions is how water is extracted from the agar. Presumably, this occurs because the osmolarity of the fluid within the swarm is higher than that within the agar.

In this work, we probed the change in osmolarity within *E. coli* swarms with osmolarity-sensitive liposomes, inspired by the work of Jayaraman *et al.*(14); see below. First we deposited liposomes in spots ~450 μm in diameter and collected fluorescence signals from entire spots as swarms ran over them. To improve the spatial resolution, we made liposome pads that were 5 times larger in diameter than the spots, allowed the swarms to run over them, and then scanned the pads with an excitation beam only 20 μm in diameter. We found that the osmolarity rises abruptly near the leading edge of the swarm, then drops to a level close to that of the virgin agar ~100 μm behind. The intervening region is one of high cell density. Evidently, growth in this region generates a substantial concentration of soluble osmolytes, which draw fluid out of the underlying agar. As the swarm expands, some of these osmolytes diffuse into the agar, raising its osmolarity above the initial value. Cells following behind the band of high cell density move over the region formerly covered by these cells and experience an osmolarity inversion that draws some fluid back into the agar. Thus, one expects to see the biphasic flow profile predicted by the fluid flow measurements. The excess fluid that remains in the swarm fuels its spreading.

MATERIALS AND METHODS

Swarm plates

Swarm agar contained 1% Bacto peptone, 0.3% beef extract, and 0.5 % NaCl (the swarm medium) and 0.48 % Eiken agar. For tests of the influence of plate osmolarity on swarming

velocity, 0.5 – 0.55% NaCl and 0.48– 0.55% Eiken agar were used. The agar was autoclaved or melted in a microwave oven and cooled to ~60°C, and then 0.5% filter-sterilized arabinose was added. 25 ml aliquots were pipetted into 150 mm dia. × 15 mm deep Petri plates. The plates were swirled gently to spread the agar over the entire plate, cooled for 15 min inside a Plexiglas box, and then inoculated.

Cell growth

Bacterial strains AW405 (15) and HCB1668 (16) were grown under conditions described previously (10). Both strains were used for preliminary work, and AW405 for the final experiments. In brief, single bacterial colonies were cultivated in LB broth overnight at 30°C and diluted 10^{-5} with swarm medium. 1 µl of this suspension was dispensed ~ 3 cm from the edge of a swarm plate, and the plate was dried in the Plexiglas box for another 30 min, until the inoculum was completely absorbed. Plates were incubated at 30°C and ~100% relative humidity.

Liposome preparation

Lipids (in chloroform) were purchased from Avanti Polar Lipids, Inc. The liposomes were prepared from 20 mg L- α -phosphatidylethanolamine (from *E. coli*), 6 mg 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000], 6 mg 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000], and 3 mg cholesterol (molar ratio of 1 : 0.04 : 0.04 : 0.3). The mixture was dried with gently flowing nitrogen gas in a rotating 50-ml round-bottom flask for 30 min followed by vacuum evaporation for 3 h. The lipid film was hydrated with 1 ml dye solution under argon with agitation at 160 rpm at room temperature for 2 h. 18.67 mg calcein (Life Technologies Corp.) and 1.21 mg sulforhodamine-101 (Life Technologies Corp.) were dissolved in Buffer I that contained 0.27

92 mM KCl, 0.147 mM KH_2PO_4 , 1.5 mM Na_2HPO_4 , 0.2 mM Tris, 102 mM NaOH, and 5 mM NaCl.

93 The final pH was adjusted to 7.4 with 1 M HCl, and the osmolarity to 240 mOsm with 1 M NaCl.

94 Osmolarity was measured with an Osmette II osmometer (Precision Systems, Inc., Natick, MA).

95 0.5-ml aliquots of the multilamellar lipid vesicles generated by this procedure were stored in

96 argon at -20°C , where they were stable for a few months; typically, we used them within one

97 month.

98 The dye-encapsulated multilamellar vesicles were converted to unilamellar vesicles by 5

99 cycles of freezing in dry ice for 5 min and thawing at 50°C for 15 min. The unilamellar vesicles

100 were then passed through a $0.8\text{-}\mu\text{m}$ Nuclepore Track-Etch membrane (Whatman) 21 times at

101 50°C with a Mini-Extruder (Avanti Polar Lipids, Inc.) that was preconditioned with Buffer I at

102 50°C for 20 min. This generates liposomes $\sim 0.5\text{ }\mu\text{m}$ in diameter. The liposomes were cooled to

103 room temperature for 5 min before loading on a Sephadex G-50 coarse gel filtration column to

104 remove free dyes. To prepare the column, the Sephadex beads were swelled with Buffer II at 4°C

105 for 2 days. Buffer II differs from Buffer I in that it contained an additional 0.5 mM CaCl_2 and 0.5

106 mM MgCl_2 . The Sephadex slurry loaded on a column ($1\text{ cm} \times 25\text{ cm}$) was washed with 4

107 volumes of swarm medium. The liposomes were eluted with the same medium (osmolarity 245

108 mOsm), and collected in glass vials. The liposomes were stable for up to a week when stored at

109 4°C under argon; typically, we used them within 3 days. About 0.5 ml liposome suspension

110 could be generated in one preparation. Aliquots were stored in 8 vials. Each vial was used no

111 more than 3 times; otherwise, a new G/R ratio osmolarity calibration was performed. The

112 liposomes were quite hardy. They did not fuse on colliding with one another. Nor did they

113 break when pipetted onto the surface of agar or when struck by swimming cells. Had they done

114 so, the dyes would have diffused into the underlying agar.

Fluorescence detection and imaging

The swarm plates were mounted on the temperature-controlled stage of an upright microscope (Nikon Optiphot2) as described in (10), except that a 20x bright-phase objective was used. The entire setup, except for the photomultiplier power supplies and computer, was enclosed by a black foamboard box. Fluorescence was excited by a cold white LED (Thorlabs) via an FITC/Texas red fluorescence cube (Chroma). The fluorescence from calcein (G) and sulforhodamine-101 (R) was detected by photon-counting photomultipliers (Hamamatsu H7421) connected to a data-acquisition board (National Instruments USB6211), and quantified with custom software written in LabView (National Instruments), which provided the G/R ratio.

10 nL liposome suspension was dispensed with a 0.5- μ L Neuros syringe (Hamilton Co., Reno, NV) and formed a spot \sim 450 μ m in diameter similar to the size of the microscope's field of excitation. Liposome spots were placed at locations ahead of the swarm according to the estimated swarm expansion rate, so that the bacteria would arrive 1.5 h later. Data acquisition was started 30 min after dispensing. The liposomes ended up on the top of the agar and were overrun by the advancing swarm.

Phase-contrast videos were recorded using a Hi-res Exvision CCD camera connected to a digital video cassette recorder before and after recording the fluorescence signal, as described in (10). Swarming speed was determined after importing the images to a computer, as described before (10). Times on the traces were then converted to distance from the swarm edge using the swarm expansion rate. To monitor the bacterial behavior on the liposome spots, continuous video clips were recorded at the proximal edge, the distal edge, and in the middle of the spots.

136 Alternatively, snapshots of a few seconds were taken during a fluorescence recording session by
137 transiently blocking the light path to the photomultipliers and activating the video system.

138 Scanning swarms moving on liposome pads

139 All scanning experiments were performed with standard swarm plates and a 40x bright-phase
140 objective. 50 nl liposome suspensions were dispensed with the Neuros syringe to form liposome
141 pads that were ~2.3 mm in diameter. The liposome pads were placed at locations where a swarm
142 would arrive in ~2 h, based on the swarm expansion rate. The liposome pads had a thick edge
143 ~100 μm wide. Only fluorescence signals from uniformly-distributed liposomes found within
144 this edge were collected. The microscopic field of excitation was reduced to a diameter of 20 μm
145 by closing the excitation beam field iris.

146 The observation field for phase contrast (~500 μm in diameter) was centered ~100 μm
147 away from the proximal edge of the liposome pad, between the pad and the advancing swarm.
148 The swarm movement was monitored with the Hi-res Exvision CCD camera, using the
149 microscope tungsten source. When the bacteria reached the liposome pad, the light path was
150 switched to the photomultipliers and fluorescence signals were recorded. After 10-40 s, the
151 swarm plate was pushed by an Intelligent Picomotor (New Focus, San Jose, CA) at 10, 20, or 30
152 $\mu\text{m/s}$, so that the excitation beam scanned the swarm in the swarm's direction of motion.
153 Scanning was continued until the excitation beam reached the far edge of the pad, and then the
154 plate was quickly pulled back manually and a 2nd scan initiated. The location of the interface
155 between the monolayer and multilayer region of the swarm – see Fig.1 of (10) – was determined
156 by briefly looking through the microscope during the scanning process. The swarming speed on
157 agar was determined from the phase contrast video taken before the fluorescence scanning. The

swarming speed on liposome spots or pads was based on videos taken on swarms that were not subjected to fluorescence recording. The traces of G/R ratios of the two scans were superimposed after determining distances from the monolayer/multilayer interface, which was taken as position 0.

Calibration curves

To convert the G/R ratio to osmolarity, we plotted two kinds of standard curves (Fig. 1). The G/R ratio in solutions of different osmolarities was determined by mixing 1 μ l liposome suspension with 44 μ l standard swarm medium or standard solution in a silicon-grease well on a microscopic slide. Two 0.46 mm spacers made from double-sided Scotch tape and the paper disks supplied with Millipore filters were placed outside the grease ring to fix its depth, and a narrow channel was included through its wall to relieve the pressure when a coverslip was added to seal the top. This channel was sealed with grease before the measurements were made. Standard swarm media were prepared by adding different amounts of NaCl to swarm media, and their osmolarities were measured with the Osmette II osmometer. Standard solutions were either purchased from Precision Systems Inc., or made by adding 1 M NaCl to Buffer I. The slide was prewarmed for 5 min at 30°C in the dark, and the fluorescence signal was recorded for 500 s at 30°C. The averaged values of G/R ratios obtained between 300 s and 350 s were used to plot the calibration curves. The part after 250 s was fitted by linear regression to determine the photo-bleaching rate.

Swarm plates with different osmolarity were prepared by adding different amounts of NaCl to the swarm agar. A 10 nL liposome suspension was dispensed on the agar plate. After 30 min incubation in the dark, fluorescence signals were recorded for 2 h. Then 0.5 mL agar was

180 scratched from the plate and centrifuged at 16,000 g for 20 min. 50 ul clean supernatant was
181 taken out for determination of the osmolarity with the Osmette II osmometer. The G/R traces
182 were then fitted by linear regression, and corrected for photo-bleaching. The curves corrected for
183 photo-bleaching were extrapolated back 30 min to obtain the values for the G/R ratios free of the
184 influence of evaporation. These values were used to plot the standard curves for liposomes on an
185 agar surface. The calibration curves of the scanning experiments were obtained with a procedure
186 similar to the real experiment, but on control plates with different osmolarities. The liposome
187 pads were equilibrated 30 min before scanning, so that dense packing of liposomes would not
188 occur.

189

RESULTS AND DISCUSSION

Osmolarity-sensitive liposomes

Liposomes were prepared by a recipe adapted from Jayarman *et al.* (14), as described in MATERIALS AND METHODS. Two kinds of fluorescent dyes were encapsulated, one that self-quenches when liposomes shrink (G, the green dye calcein, emitting at 519 nm) and the other that does not (R, the red dye sulforhodamine-101, emitting at 619 nm). Therefore, the G/R ratio reflects the osmolarity of the surrounding fluid. The lipid composition of the liposomes described by Jayarman *et al.* was not well defined, and the liposome response curves were non-linear (14). We tested different combinations of diacyl glycerophospholipids and their polyethylene glycol-modified derivatives. Liposomes made from mixtures of *E. coli* L- α -phosphatidylethanolamine, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000], 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-5000], and cholesterol worked well at 30°C. They responded to the change in osmolarity linearly over a range of about 250 mOsm, as shown in Fig. 1C. We prepared the liposomes at 245 mOsm, so they could be used to measure up to 500 mOsm. These liposomes were stable up to a week when stored in argon at 4°C and up to 5 h when applied to a swarm plate in air at 30°C.

Since we used photon counting, the intensity of the excitation light was low, and the effect of photobleaching was small (Fig. 1A). Photobleaching was estimated by fitting the G/R ratio recorded in liquid suspension by linear regression. The data for the first 250 s were excluded to allow time for equilibration. The averaged slope of the fitting curves was $(3.75 \pm 0.7) \times 10^{-6}$ per second (mean \pm SD, $n = 9$). This amount of photobleaching leads to a small correction.

on all measured curves. The G/R ratios recorded between 300 s and 350 s were averaged for plotting the standard curves in suspension (Fig. 1C); within this short time photo-bleaching was small enough to be ignored.

To obtain the calibration curves on plates, the G/R values were corrected for photo-bleaching and then extrapolated to the time of dispensing, so that evaporation through our temperature-control apparatus could be ignored (Fig. 1B). The calibration curve thus generated had a steeper slope than that found for liposomes in suspension (Fig. 1D). The two curves crossed at the point where the osmolarity of the agar matched that of the medium in which the liposomes were prepared. The liposomes form a multilayer disk on the agar surface. It is likely that if the agar surface and the liposomes are isotonic, the liposomes remain spherical and the inter-liposome space is similar to that in suspension; while on an agar surface that is hypertonic, the shrunken liposomes are more densely packed, like flattened balls. If so, the loss of light by absorption or diffraction might be more severe in the green than in the red, leading to a decrease in the G/R ratio. Whatever the reason, liposome packing caused a reduction of signal equal to 6.42 mOsm per 0.01 G/R ratio. The calibration curves for the scanning experiments were obtained with liposome pads made from larger aliquots of liposomes equilibrated for shorter periods of time, before dense packing became significant, so these curves were similar to those obtained with liposomes in suspension.

Monitoring osmolarity in swarms

We first tried to monitor the change in osmolarity in real time, by collecting fluorescence signals from a fully illuminated fixed liposome spot $\sim 450 \mu\text{m}$ in diameter, as an advancing swarm ran over it (Fig. 2). Although we used the same swarm medium to prepare the plates and the

liposomes, the medium in the liposome suspension needed more than 1 h to equilibrate with the medium in the agar. After equilibrating >1.5 h, traces of G/R ratios showed a quick decrease upon the arrival of bacteria, followed by slow recovery to a level slightly higher than the baseline before the invasion (Fig. 2B, with the long-time end of the trace not shown). In cases when swarms expanded rapidly, traces recorded after waiting for >1.0 h were occasionally used to calculate the changes in osmolarity.

At the beginning of each trace, there was a section where the fluorescence signals were recorded before the arrival of the swarm (Fig. 2). These signals provided a baseline for subsequent changes in osmolarity. The calibration curve measured on plates (Fig. 1D, black open diamonds) was applied. Extrapolating the baseline to the time when the liposomes were dispensed allowed us to estimate the osmolarity that supports active swarming under our experimental conditions (~296.8 - 316.2 mOsm). By changing the concentrations of NaCl and Eiken agar, we could obtain swarms that expanded at velocities ranging from 1.0 $\mu\text{m/s}$ to 4.3 $\mu\text{m/s}$ (Fig. S1 in the Supporting Material). There was an inverse correlation between swarming speed and agar osmolarity.

There was a transient increase of both the green and red signals when the swarm invaded the liposome spot (Fig. 2A). The swarm drew water out of the agar and lifted the liposomes from the packed state to the suspended state: the motionless liposomes began jiggling around when the bacteria arrived. This process suddenly increased the inter-liposome space, decreasing the loss of light by absorption or diffraction. Thereafter, a small amount of liposomes were carried away by the swarm fluid, causing a slow decrease of both fluorescence signals, but this process would not change the G/R ratio. It is the decrease of green fluorescence caused by self-quenching that is responsible for the persistent change of the G/R ratio (Fig. 2B).

257 After bacterial invasion, the calibration curve measured on agar is only applicable to
 258 some liposomes within the excitation field, while the curve in suspension would be applicable to
 259 the others. The measurements were corrected for the fraction of liposomes in the packed state vs.
 260 those in suspension, and the *bona fide* osmolarity was plotted in Fig. 2C. The distance inside the
 261 swarm (negative towards the swarm center) was determined by multiplying the time after
 262 invasion by the instantaneous velocity of the swarm front. The location of the leading edge of
 263 the swarms was at position 0.

264 Before the large increase of osmolarity shown in Fig. 2C, a small peak of width $51.0 \pm$
 265 $18.3 \mu\text{m}$ ($n=6$) always appeared (Fig. 2D, grey arrows). It was followed by a brief, sharp
 266 biphasic dip with recovery. We believe this peak was due to the arrival of the stream of fluid
 267 that flows in a clockwise sense in front of the swarm (12); see Fig. S2A. This small amount of
 268 fluid caused an increase of osmolarity $2.8 \pm 0.9 \text{ mOsm}$. Within $\sim 10 \text{ s}$ the bacteria arrived, and a
 269 large amount of fluid flooded the spot.

270 The actively-expanding rim of a swarm can be divided into four regions (10, 11). The
 271 outermost region is a monolayer of cells, many of which are stuck on the agar surface. These
 272 cells are released when the cells of the second multilayer region, which are vigorously motile and
 273 at high density, catch up. The cell density decreases in the third region, dubbed the falloff,
 274 leading to the plateau, where motility remains at a relatively low level. When the monolayer
 275 cells (and some from groups in the high-density region) rushed onto the liposome spot (Fig.
 276 S2A), the cells dispersed into a band $130\sim 150 \mu\text{m}$ wide, and the cell density decreased from
 277 $\sim 0.18 \text{ cells}/\mu\text{m}^2$, as in the normal swarm monolayer, to $\sim 0.03 \text{ cells}/\mu\text{m}^2$. This dispersed
 278 monolayer contained the same number of cells, but covered 4.5 times as large an area as the

monolayer on agar. The front edge of this dispersed monolayer was irregular in shape, with dynamic protrusions and invaginations.

The osmolarity increased steadily upon the arrival of the cells, and then the rate of increase slowed down, until an osmolarity maximum was reached (Fig. 2 D). The rapid increase took about ~35s, while the slow increase that followed was swarm-speed dependent. The whole process of this osmolarity jump took place within a distance slightly longer than the diameter of the liposome spot, $480 \pm 22 \mu\text{m}$ ($n=6$). The dispersed monolayer and multilayer regions moved rapidly forward at $6.0 \pm 1.0 \mu\text{m/s}$ ($n=5$) until the dispersed monolayer reached the far edge of the liposome spot. The osmolarity jump – the difference between the osmolarity maxima and the baseline (Fig. 2C, double-headed arrow) – was $11.4 \pm 5.0 \text{ mOsm}$ ($n=15$). The steady level reached at the end of the experiment was $3.7 \pm 0.6 \text{ mOsm}$ ($n=15$) higher than the baseline. This value reflects the osmolarity of the swarm interior. When the monolayer cells reached the far edge of the liposome spot, the cells stalled and the multilayer caught up with decreasing velocity (Fig. S2A). Eventually, the swarm ran over the far edge of the liposome spot and continued advancing on the agar. Because the swarm expanded faster on the liposome spot than on the adjacent agar, a hump formed at the swarm front that eventually smoothed out.

Scanning across swarms moving on liposome pads

The fixed spots did not provide the spatial resolution required to evaluate the fluid-flow model (10). Therefore, we performed another set of experiments in which the liposome pads were 5 times larger in diameter (Fig. 3). The excitation beam, $20 \mu\text{m}$ in diameter (not shown), was held fixed, while the plate was pushed by a picomotor, so that the swarm was scanned in its direction of motion, as shown by the small arrows in Fig. 3. The fast expansion rate of the swarm on the

301 liposome pad made the swarm spread out, so that the monolayer and multilayer regions of the
302 swarms were cleanly separated from their counterparts on the agar surface (Fig. 3). Because the
303 front of the dispersed monolayer was not uniform, we used the position of the
304 monolayer/multilayer interface as our reference point (position 0).

305 To convert measurement time to distance, the speed of the excitation beam relative to the
306 reference origin is required. To a first approximation, this is just the swarm speed less the plate
307 pushing speed. For a second scan performed on the same plate, the swarm speed decreased as
308 the dispersed monolayer approached the far end of the pad, so we linearly interpolated the swarm
309 speed from 6 $\mu\text{m/s}$ to the respective swarm expansion rate on agar. The resulting osmolarity vs.
310 distance plots are shown in Fig. 4A.

311 The scanning experiment revealed that the multilayer region of the swarm contains two
312 distinct bands, an outer high-osmolarity band of 327.1 ± 6.5 mOsm, and an inner low-osmolarity
313 band of 302.5 ± 5.0 mOsm (Fig. 4A). The osmolarity baseline on the agar surface measured with
314 the fixed liposome spot under identical conditions was 302.1 ± 2.3 mOsm ($n=8$) (Fig 4A, dark
315 and light orange lines). The outer band is therefore 25.3 ± 7.3 mOsm higher than the baseline
316 and the inner band is almost isotonic to the agar. Farther inside there is a stable plateau that is
317 11.3 ± 3.1 mOsm higher than the baseline. The osmolarity eventually went down to the interior
318 level that was 3.7 ± 2.9 mOsm above the baseline. The dispersed monolayer, a band ~ 150 μm
319 wide to the right of position 0, had an osmolarity 9.2 ± 5.3 mOsm above the baseline.

320 The osmolarity profile inside a bacterial swarm

321 The liposomes were covered with polyethylene glycol to increase stability, which has certain
322 surface properties (e.g., hydrophilicity) that make swarms expand faster on liposome spots or

pads than on virgin agar. In the scanning experiments, different regions of the swarm were fully expanded and well separated from their counterparts on agar (Fig. 3), but on the fixed spot, such expansion was restricted (Fig. S2B). As a result, we did not see the low-osmolarity band on spots: the low-osmolarity band is obvious in Fig. 4A but not in Fig 2D. Part of the problem was the low spatial resolution of the fixed-spot method; for example, if the leading edge of a swarm advances 50 μm over a spot of diameter 450 μm , the swarm will cover only 6% of the area of the spot, and 94% of the light reaching the detectors will come from the cell-free region.

The fixed-spot measurements generated an osmolarity increment of 2.8 mOsm when the fluid that flows in front of the swarms drained into the liposome spots. This is an underestimate because, as noted above, most liposomes in the spots were not influenced by this small amount of fluid. The experimental value was corrected by multiplying by the ratio of the area of the whole spot to the area of the segment that was wetted by the flow. The height of this segment was obtained by assuming that the width of the flow on agar was expanded 4.5 times. The corrected osmolarity was as high as the plateau (12.1 mOsm), as shown in Fig 4B.

To find the dimensions of swarms on agar from the dimensions on liposome pads (Fig. 3), we multiplied the dimensions on pads by 2.6/6.0, the ratio of the speeds on agar, $2.6 \pm 1.0 \mu\text{m/s}$ ($n=15$), to those on pads, $6.0 \pm 1.0 \mu\text{m/s}$ ($n=5$). The monolayer thus converted spanned 66 μm . The width of the monolayer in a normal swarm measures 31 μm , on average (10). To compare with the previous results, we used this smaller width and set the front of the monolayer as the reference origin (Fig. 4B). The osmolarity of the monolayer was scaled using the dilution factor of 4.5 mentioned above. This assumes that the fluid and the cells were diluted to the same degree, which would be the case if the film of fluid expanded to its original thickness. We note

345 that the cells at the outer edge of the monolayer were transiently stuck to the agar, while those in
 346 the multilayer were actively swimming (10, 11). The expanded multilayer on pads was filled by
 347 cells that flowed in from the sides and thus maintained its thickness.

348 Mapping the fluid-flow pattern inside swarms with micron-sized air bubbles (10)
 349 revealed that swarm fluid flows inwards from the edge of the swarm toward the center, while
 350 beginning from $\sim 300 \mu\text{m}$ inside the swarm, it flows outwards. Fluid balance requires that water
 351 moves out of the agar and into the swarm within a region centered $\sim 30 \mu\text{m}$ from the edge of the
 352 swarm, with a peak at the monolayer/multilayer interface. After correcting for differences in
 353 migration rates on pads vs. those on virgin agar, the osmolarity profile agreed well with the
 354 model prediction (Fig. 4B). The osmolarity increased rapidly at the monolayer/multilayer
 355 interface and reached the highest value at $\sim 30 \mu\text{m}$ inside the swarm. The osmolarity profile for
 356 the first $\sim 130 \mu\text{m}$ paralleled the surface cell-density measured earlier (Fig. 5 of ref. 10).

357 Fluid balance also required that the agar absorb water from the swarm in a region
 358 centered $\sim 120 \mu\text{m}$ from the edge of the swarm. The lowest point on our curve was at $-128 \mu\text{m}$,
 359 and it reached the baseline or slightly lower, within the error range of our measurements (Fig. 4A,
 360 light orange dotted line). The osmolarity reached a stable plateau at $\sim 200 \mu\text{m}$ from the edge of
 361 the swarm (Fig. 4B), also in accordance with the model prediction (10). The maxima obtained in
 362 the fixed-spot measurements corresponded to the plateau detected by scanning. It was ~ 11.3
 363 mOsm higher than the baseline as revealed by both methods. This plateau extended to $\sim 300 \mu\text{m}$
 364 inside the swarm. The osmolarity began to decrease beyond that point and reached the level for
 365 the swam interior $\sim 500 \mu\text{m}$ from the edge of the swarm. Active fluid flow was not observed
 366 beyond $300 \mu\text{m}$ from edge of the swarm (10), suggesting that this region of the swarm is in
 367 equilibrium with the agar.

Properties of osmolytes

The primary osmolyte that has been implicated in the swarming of *E. coli* or *Salmonella* is lipopolysaccharide (17, 18). Other candidates of high molecular weight include enterobacterial common antigen and colanic acid (18). Mutants of *Salmonella* that rotate their flagella exclusively clockwise or exclusively counterclockwise fail to swarm, yielding plates that are relatively dry (19). However, revertants that remain nonchemotactic yet frequently switch the direction of flagellar rotation do swarm, leading to the suggestion that erratically-moving flagella strip lipopolysaccharide off of the cell surface (20).

We need osmolytes of relatively high molecular weight (with relatively small diffusion coefficients) to explain our results. Substances of low molecular weight, like salts, acetate, glutamate, proline, glycine betaine, or trehalose, will not do. The swarm fluid is only a few μm thick, while the underlying agar is $\sim 1,400 \mu\text{m}$ deep. Both are nearly 100% water. A small molecule with diffusion coefficient, $D \sim 10^3 \mu\text{m}^2/\text{s}$ ($10^{-5} \text{cm}^2/\text{s}$) will diffuse $(2Dt)^{1/2} \sim 2 \mu\text{m}$ in $\sim 0.002 \text{ s}$, $20 \mu\text{m}$ in $\sim 0.2 \text{ s}$, and $200 \mu\text{m}$ in $\sim 20 \text{ s}$. The time scale of interest (Fig. 4B) is about 50 s (a swarm displaced $\sim 130 \mu\text{m}$ at the rate of $2.6 \mu\text{m}/\text{s}$). In that interval, substances of low molecular weight will be diluted by a factor of more than 100 by diffusion perpendicular to the surface of the plate, and peaks and troughs, such as those apparent in Fig. 4B, will be washed out by diffusion in a direction parallel to the surface of the plate. But substances 100 times larger with diffusion coefficients 100 times smaller will fit the bill. A substance of this size can diffuse out of the swarm fluid into the agar (neglecting opposing fluid flow) in about 0.2 s , or into the agar a distance 10 times as far, in about 20 s . So once the bulk flow subsides, the osmolyte will move into the agar. Thus, one expects a concentration inversion when the measured value of the osmotic pressure falls to the baseline, as it does in Fig. 4B. When this happens, some fluid will

391 flow from the swarm back into the agar. So the general features of our osmolarity measurements
392 are consistent with the predictions of the fluid-flow model. If the osmolytes are polyelectrolytic,
393 counter-ions will contribute to the osmolarity. We do not know whether the fall in concentration
394 of osmolytes is precipitated simply by a decreased rate of cell growth (10) or whether the
395 osmolytes are actively resorbed.

396 If the swarm spreads at 2.6 $\mu\text{m/s}$, the peak in osmolarity shown in Fig. 4B extending from
397 0 to 520 μm can be scanned in 200 s. We let the concentration of osmolyte at the surface of the
398 agar vary in time according to the output of this scan and follow the concentration of the secreted
399 osmolyte as it diffuses into the agar. We ask, as a function of the value of the diffusion
400 coefficient, D , how long it takes before the gradient normal to the surface of the agar changes
401 sign. This time interval would equal that measured earlier (10) before fluid begins to flow from
402 the swarm back into the agar, 41 ± 7 s. The acceptable range of diffusion coefficients proves to
403 be 0.7 to 6.8 $\mu\text{m}^2/\text{s}$, which is in the ballpark predicted by our order of magnitude arguments. A
404 diffusion coefficient in this range (1.1 $\mu\text{m}^2/\text{s}$) has been measured for aggregates of phenol-
405 extracted lipopolysaccharide of molecular weight $\sim 2 \times 10^8$ (21). So lipopolysaccharide is an
406 attractive candidate.

407 Conclusion

408 We optimized the liposome sensor and used it to monitor the osmolality of bacterial swarms in
409 real time. The result revealed a well-defined osmolality profile inside the bacterial swarm. The
410 flow pattern of *E. coli* swarm fluid previously described was explained. The diffusion
411 coefficient of the potential osmolyte(s) was predicted based on our model. The chemical
412 structure of the osmolyte(s) remains a question for future investigation; although,

lipopolysaccharide is a reasonable candidate. This technique is highly reproducible when applied to *E. coli*, which does not produce surfactants. The method can be applied to surfactant-producing bacteria as well, provided that the surfactants are not strong enough to destroy the liposomes. We look forward to the application of this technique in other swarming bacteria and in other research fields.

Acknowledgments

This research was supported by NIH Grant AI100902. Author contributions: LP, YW, JT, and HB designed the research; HB assembled the microscope; YW did preliminary work on the liposome prep; YW and GH did preliminary work on the fluorescence measurements; GH perfected the data-acquisition system; JT helped with the scanning experiments; LP did the final measurements and analyzed the data; LP and HB wrote the paper, with input from the other authors. The authors declare no conflict of interest.

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FIGURE LEGENDS

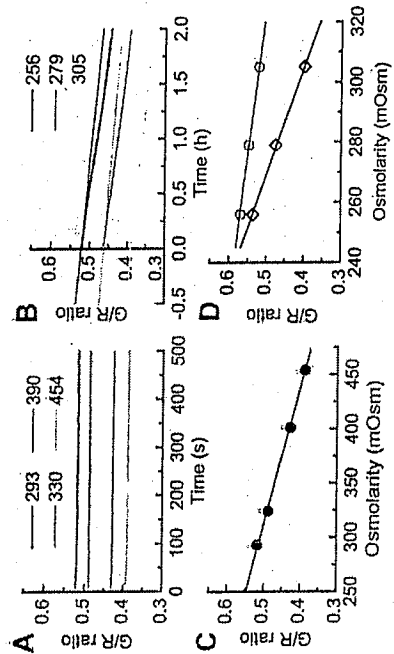
Fig. 1. Calibration of osmolarity-sensitive liposomes. **A**, the G/R fluorescence ratio recorded over an interval of 500 s when liposomes prepared at 245 mOsm were suspended in swarm media at the osmolarities indicated on the plot. Note the minimal effect of fluorescence bleaching on the G/R ratio. **B**, G/R ratios from liposomes that were loaded on agar surfaces 0.5 h before recording. Osmolarities of the swarm media used to prepare the plates were measured after the media and agar were separated by centrifugation. These values are shown on the plot. The dotted lines show the data after correction for photo-bleaching. The changes that remain are due to evaporation. **C**, G/R ratios at medium osmolarities recorded between 300 and 350 s. **D**, Comparison of the G/R ratios measured in suspension (red circles) to those measured on an agar surface (black diamonds) as a function of the osmolarity of the swarm media.

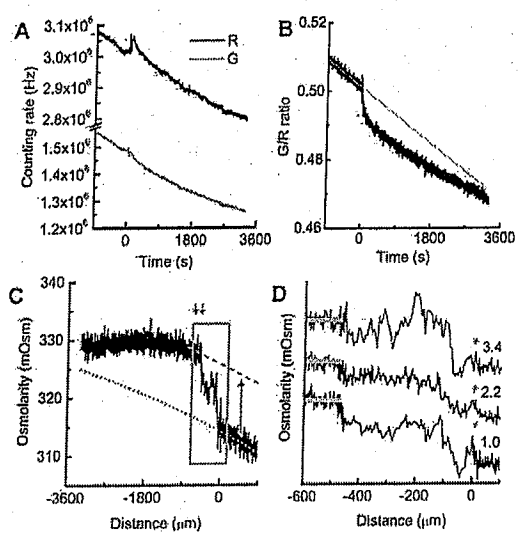
Fig. 2. Change of osmolarity on swarm plates measured with fixed liposome spots. **A**, The fluorescence signals of sulforhodamine 101 (R) and calcein (G) recorded on a swarm. Time zero corresponds to the time when bacteria first invaded the liposome spot. **B**, The G/R ratio after correction for photo-bleaching. The grey dotted line is the reference baseline for osmolarity on the agar surface, which changed because of evaporation. The time required for the measurement, set by the dimensions of the swarm divided by its spreading rate, was relatively long. **C**, The trace after the G/R ratio was converted to osmolarity and time was converted to distance (negative toward the swarm center). The grey dotted line is the reference baseline. It curved down because the evaporation rate was constant, but the swarming speed slowed down. The initial swarming speed was 1.0 $\mu\text{m/s}$. The grey broken line indicates the osmolarity maximum, and the double-headed arrow the difference in osmolarity between the maximum and the baseline. The difference between the osmolarity trace and the baseline was maximum between

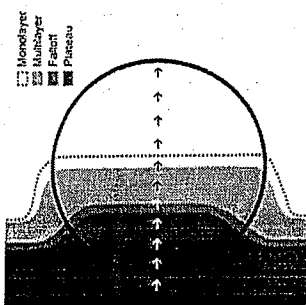
the two grey down-pointing arrows. **D**, An enlarged view of the boxed region in **C** with two other traces aligned on top of it (with swarm speeds in $\mu\text{m/s}$ noted at the right ends of the traces). The small peak at time zero is indicated by grey arrows. The osmolarity maxima are highlighted by grey bars superimposed on the traces.

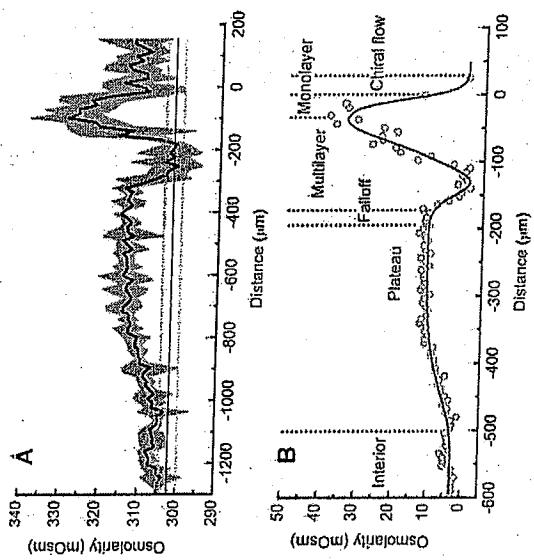
Fig. 3. A diagram showing a swarm that has arrived at the center of a liposome pad, 2.3 mm in diameter. Different grey-scale values represent different swarm regions, as labeled in the figure. The black dotted line indicates the swarm front. Arrows show the direction of the scan.

Fig. 4. Change of osmolarity inside swarms spreading on liposome pads revealed by scanning. Position 0 corresponds to the monolayer/multilayer interface. Negative values are toward the center of the swarms. **A**, Means (black line) and standard deviations (grey areas) from 9 scans on 7 different plates. The osmolarity baseline measured on the agar surface is shown by a dark orange line, and its standard deviation by light orange dotted lines. Note that each scan was completed in less than 2 min with liposomes submerged in swarm fluid, so evaporation was not a problem. **B**, The osmolarity profile inside a swarm after subtraction of the baseline value for virgin agar and conversion of the distance on the liposome pad to that on agar. The grey open circles are measured values and the black line is the curve that fits the data. The monolayer was normalized (shrunk to normal size) and the reference point was set to the front. The osmolarity values were corrected for dilution that occurred when the swarms spread over the liposome pads. The notations monolayer, multilayer, falloff, and plateau refer to regions of different surface cell-density described in ref. 11.









The NIHMS has received the file 'mmc1.pdf' as supplementary data. The file will not appear in this PDF Receipt, but it will be linked to the web version of your manuscript.

Supporting Material

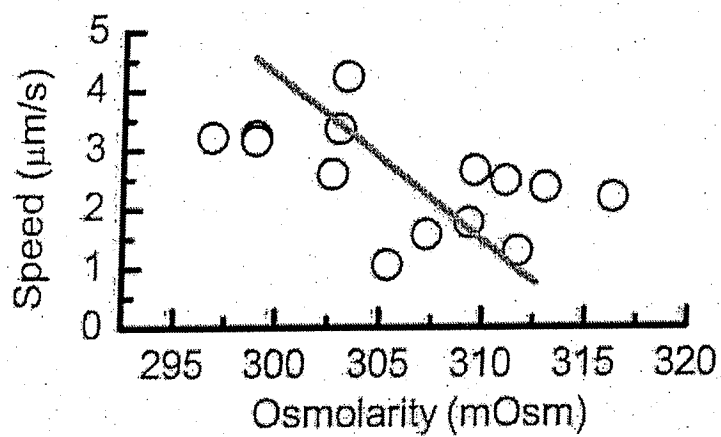


Fig. S1. Dependence of initial swarm speed on agar osmolarity, with a linear fit.

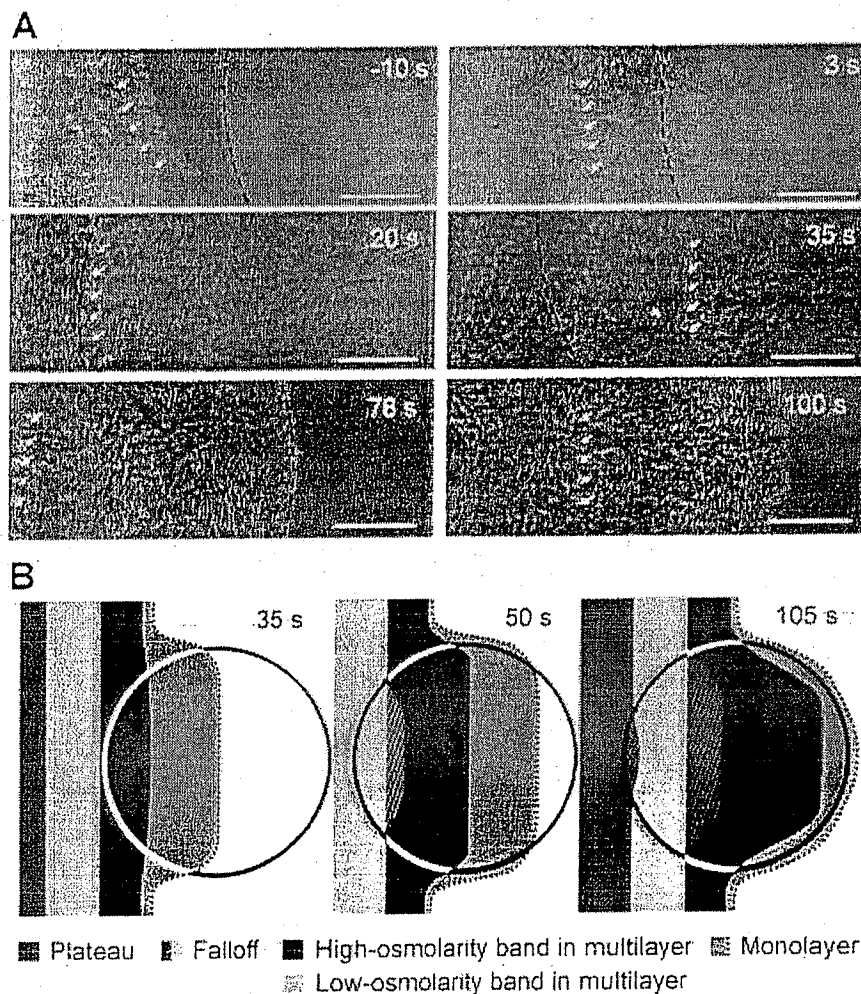


Fig. S2. Interaction between bacteria and a fixed liposome spot. **A**, Snapshots of swarms running over liposome spots taken from three different movie clips. Broken arches depict the proximal or distal edges of the liposome spots. The chiral flow in front of the swarm appears as slightly dark shade. Scale bars equal 50 μm . Approximate times relative to the initial time of invasion are shown on the top-right corner. White arrows highlight the monolayer/multilayer interfaces. **B**, Diagrams showing the movement of a swarm on liposome spots at different times. Grey levels correspond to cell densities and osmolarities of different regions of the swarm, as indicated in the bottom legend. The black dotted line is the swarm front. Shading indicates the minimal area where cells from swarm regions might have mixed.